that the toxins can be used safely for vaccines because the effective concentration of adjuvant toxins is sufficiently below the level where the toxins exhibit toxicity to humans and other animals. Thus, the present inventors studied the possible problems associated with repeated administration of the adjuvanted intranasal vaccine and reported the result previously (S. Tamura et al., Vaccine 15, 1784-1790, 1997). Nonetheless, many experts have expressed concerns about safety of toxin-adjuvant vaccine due to, for example, the difference of susceptibility to cholera toxin between human and experimental animals as well as the possibility of causing diarrhea (M.M. Levine et al. Microb. Rev. 47, 510-550, 1983).

The following methods have been proposed in order to take advantage of excellent activity of bacterial toxin in enhancing immunity and simultaneously to solve the problem of safety. However, none of the methods come close to reaching the goal of realizing both safety and adjuvant activity.

(1) Attenuation by chemical or physical treatment:

A method to reduce the activity of toxin without losing the adjuvant activity - wherein the toxin is treated with formalin, glutaraldehyde, acid, alkali, etc. or treated at a temperature severe for the toxin. For example, it is known that cholera toxin treated with glutaraldehyde has a toxic activity reduced to about 1/1000 as compared with the natural one while maintaining adjuvant activity (X. Liang et al., J. Immunol. 143, 484-490, 1989). However, toxin activity reduced to 1/1000 relative to that of the natural toxin is evaluated not to be sufficiently safe.

(2) Search for cross-reacting mutant proteins produced by mutant strains:

A method for searching cross-reacting mutant proteins of interest which retain adjuvant activity - wherein toxin-producing bacteria are treated with a mutagenizing chemical agent and toxins produced by resulting mutant strains are searched to find proteins of interest. For example, a cross-reacting protein (cross-reacting material; CRM), which showed low toxic activity and was reactive to anti-pertussis toxin antibody, was found in culture medium of a pertussis toxin-producing bacterial mutant strain. This mutant toxin is

immunogenic and can be a candidate for the pertussis vaccine antigen (Y. Sato et al., Dev. Biol. Stand. 73, 93-107, 1991). In addition to this, there is a possibility that the mutant toxin might be used as the adjuvant with low toxic activity. However, the degree of toxic activity and adjuvant activity of the mutant toxin has not been reported.

Cross-reacting protein of diphtheria toxin, CRM-197 (DT-G52E) (T. Uchida et al., J. Biol. Chem. 248, 3838-3844, 1973; G. Giannini et al., Nucleic Acids Res. 12, 4063-4069, 1984), and a cross-reacting protein of pseudomonad enterotoxin A, CRM-66 (H462Y) (S.J. Cryz et al., Rev. Infec. Dis. 5 Suppl. 5, S992-S997, 1983; S. P. Kessler et al., J. Biol. Chem. 267, 19107-19111, 1992) were reported as mutant toxins which substantially lack ADP-ribosyltransferase activity. In these mutant toxins, the ADP-ribosyltransferase activity was used as the only marker for the toxic activity of these mutant toxins, and thus there is no denying of the existence of residual toxic activity generated by other mechanisms. Thus, it is possible that these mutants have other toxin activities. In addition, it was reported that when chemically linked to a polysaccharide antigen, although having adjuvant activity, CRM-197 (DT-G52E) did not stimulate sufficient antibody titers, and that consequently it required the use of additional second adjuvant (G. S. Bixler et al., Adv. Exp. Med. Biol. 303, 185-190, 1991; D. M. Granoff et al., Vaccine 11 Suppl. 1, S46-S51, 1993).

(3) Preparation of attenuated mutant toxins by genetic engineering:

A method for constructing recombinant toxins, which still retain adjuvant activity, - wherein the toxic activity is reduced by artificially altering one or more amino acid residues of the toxin molecule utilizing gene recombination techniques. As examples, recombinants of *E. coli* heat-labile toxin, wherein the arginine residue (Arg/R) at amino acid position 7 or 192 from the N-terminus was substituted with a lysine residue (Lys/K) (LT-R7K, M.T. De Magistris et al., Dev. Biol. Stand. 92, 123-126, 1998) or with glycine residue (Gly/G) (LT-R192G, B. L. Dickinson et al., Infection Immun. 63, 1617-1623, 1995), were reported to substantially lack the toxic activity but still retain the adjuvant activity.

However, according to experiments of an other research group,

the toxic activity was not negligible in the above-mentioned recombinant toxins. Further, the recombinant proteins were unstable as compared with the natural one. As a result, larger amounts of the recombinant protein (relative to that of the natural one) must be used to raise antibody titer to the same level as that given by the natural one (K. Komase et al., Vaccine 16, 248-254, 1998; C. C. R. Grant et al., Infect. Immun. 62, 4270-4278, 1994).

When a toxic activity is caused by multiple activities, the reduction of any one of the activities can result in the manifestation of the other activities. The attenuation of toxin can be associated with this type of phenomenon, and therefore the marker of toxic activity should be selected carefully. As described above, attenuated adjuvants, which have been reported so far to exhibit very low toxicity, still have problems to be solved in regard to adjuvant activity, safety, stability, and productivity. Further, many screening steps must be repeated to achieve reduction of toxic activity and preservation of adjuvant activity, which are contrary subjects, by amino acid substitution. Therefore, it is a time-consuming work.

Disclosure of the Invention

An object of the present invention is to provide an attenuated toxin, that has substantially no toxic activity but still has the excellent adjuvant activity of the toxin, as a new adjuvant.

Another object of the present invention is to provide a vaccine using one or more adjuvants of the present invention, that is safe and effective and that can be obtained by relatively simple and convenient procedures. More specifically, the object is to provide a vaccine that can be used in reduced doses, or can be used without loss of its immunogenic activity when administered by a vaccination route other than injection.

The present inventors have studied methods to reduce the toxic activity of a variety of toxins and found out that an attenuated toxin which can be prepared by a chemical treatment has substantially no toxic activity, but still has sufficient adjuvant activity of the levels to meet the criteria of practical application. The present inventors have further verified that high level of safety is expectable